metastases ($p < 0.0018$), which did not differ statistically from either v. MCB7-2/β-gal or v. MCB7-1/B7-2/β-gal. Adjuvant IL-2 did not statistically improve upon these results.

No statistical difference between rVV expressing either B7-1, B7-2, or both could be demonstrated. Of note, however, there is some suggestion that B7-1 may be a more potent costimulator of antitumor immunity than B7-2. That is, rVV expressing B7-1 alone mediated a 10 fold greater reduction than a rVV expressing B7-2 alone, or both B7-1 and B7-2. Similarly, rVV expressing both B7-1 and B7-2 was three times as potent as the vector containing the irrelevant protein, MHA together with B7-2. However, whereas the B7-1 and B7-2 molecules are within different regions of the VV genome, under independent promoters, whether these findings are reflective of an increased potency for B7-1 as a costimulator, or merely reflective of variable expression will require further analysis.

Example 25

Effect of In Vivo T cell Subset Depletions on the Ability of v. MCB7-1/β-gal to Mediate Tumor Regression of Established Disease

Adoptive transfer and active immunotherapy studies demonstrated a specificity of response suggesting that the cell-mediated arm of the immune system, i.e., T-lymphocytes, were responsible for tumor regression. To specifically identify the T cell subset which is responsible for the cytotoxic antitumor response observed, we repeated three-day active treatment study while selectively depleting either CD4+ or CD8+ cells with anti-CD4 and anti-CD8 antibodies. In mice treated with the anti-CD4+ antibody FACS analysis revealed > 99.8% depletion of CD4 cells, whereas in mice treated with the anti-CD8+ antibody, >97% depletion of CD8+ was seen (Figs. 21a-21f). These results were confirmed in three other

FACS analysis completed 1 day before and seven days after treatment. In the groups immunized with HBSS, no tumor regression was seen (Fig. 21g). In groups immunized with v. MCB7-1/b-gal, both the no depletion group and the CD8+ depletion group showed a significant reduction in the number of pulmonary metastases ($p < 0.005$, and $p < 0.054$, respectively). The mean reduction in the no depletion group was approximately 10 fold greater than that seen in the CD8+ depleted group. Notably, in mice depleted of CD4+ cells, there was no tumor regression seen. This suggests a critical role of CD4+ cells in mediating this cellular immune phenomenon. Similarly, in the v. MCMHA/b-gal immunized group, no reduction in the number of metastases was seen in the CD4+ depletion group, yet a small but significant reduction was seen in both the no depletion group, and the CD8+ depletion group ($p < 0.02$, and $p < 0.06$, respectively) (data not shown). Mice immunized with v. MCB7-1/NP showed no reduction in the number of pulmonary metastases in any group (data not shown). These results demonstrate that the reduction in pulmonary metastases is T cell mediated, and that both CD8+ and CD4+ lymphocytes are required to mediate this phenomenon. These results are contrary to most transfection studies in which a cytotoxic CTL response was induced in the absence of CD4+ cells. When the genes for B7-1 or B7-2 are transfected into tumor cells, it is the tumor cells themselves which act to present a TAA in the context of MHC and B7 to responding T cell. Following vaccination with a rVV coexpressing both B7 and the TAA, all infected cells expressing MHC class I molecules would theoretically function to activate T cells. In the transfection studies, CD+ 8 depletion abrogated the antitumor immune response, by removing the responding cytotoxic population. Following v. MCB7-1/b-gal vaccination however, although CD8+ depletion would significantly reduce the precursor frequency of responding

cytotoxic cells (3% remained in this study), the increased number of cells capable of presenting antigen and costimulating may blunt the effect of the depletion. Thus, the incompleteness of the CD8+ depletion may be a simple explanation for the observed results. However, the importance of CD4+ cells in providing necessary help to proliferate a CTL response in this model system cannot be minimized. CD4+ depletion completely abrogated any tumor regression following vaccination. Whether this phenomenon is mediated simply by a reduction in IL-2 production normally provided by CD4+ T helper cells, or represents a depletion of either Th1 or Th2 cells important in mediating the antitumor response is unclear.

Example 26

Materials and Methods for IL-2 Enhancement of Recombinant Poxvirus-Based Pulmonary Metastases

Cell Lines

CT26 is an N-nitroso-N-metylurethane-indiced BALB/c ($H\text{-}2^d$) undifferentiated colon carcinoma generously supplied by D. Pardoll (Baltimore, MD). CT26 was cloned to produce a wild-type parental tumor line, CT26.WT. The gene for *lacZ* was stably transfected into CT26.WT as described previously (Wang et al, J. Immunol. 154:4685, 1995). Briefly, a plasmid donated from A.D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA) containing the gene for β-gal and a neo-resistance marker were used to construct an ecotropic producer cell line secreting the LZSN retroviral construct. This retroviral supernatant was used to transduce the CT26.WT cell line. Transductants were selected in a G418 media and then subcloned by limiting dilution analysis at 0.3 cells/well. Subclones that expressed β-gal were screened by X-gal staining and in ^{51}Cr release assays with anti-β-gal effectors. The subclone CT26.CL25 was selected for use in all studies because of its stable expression of both β-gal and the class I molecule H-2 L^d. A clone of the mouse

thymoma EL4 (H-2^b) stably transfected with β-gal, termed E22 (provided by Y. Paterson, (Department of Microbiology, University of Pennsylvania, Philadelphia) was used as a negative control in ^{51}Cr release assays. BSC-1 cells (American Type Culture Collection (ATCC), Rockville, MD; CCL 26) were used to expand and titer all VV. Cell lines were maintained in RPMI 1640, 10% heat-inactivated FCS (Biofluids, Rockville, MD), 0.03% L-glutamine, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 50 μg/ml gentamicin sulfate (National Institutes of Health Media Center, Bethesda, MD). CT26.CL25 and E22 were maintained in media containing 400 μg/ml G418 (Life Technologies, Inc. Grand Island, NY).

rVV and rFPV

All the rVV used in this study were originated by insertion of the foreign genes into the VV thymidine kinase (TK) gene by homologous recombination, resulting in the generation of TK-negative progeny as described (Chakrabarti et al, Mol. Cell Biol. 5:3403, 1985). The recombinant stocks were produced by using the TK-human osteosarcoma 143/B cell line (ATCC, CRL8303). From these stocks, rVV were propagated in BSC-1 cells and used as crude cell lysate. The BSC-1 cell line was also utilized to determine virus concentration by plaque titration. The rVVV used in a single experiment were titered together to maximize the accuracy of the relative titers. Preparation of rVV expressing the influenze A/PR/8/34 nucleoprotein (NP) was previously described (Smith, G.L. et al Virology 160:336, 1987). In the HPV16-E6 Vac, *Escherichia coli lacZ* was under the control of the early promoter element of the VV $p_{7.5}$ promoter from plasmid pSC65 (S. Chakrabarti, J. Sisler, and B. Moss, NIAID, NIH, Bethesda, MD); this construct was named VJS6 for simplicity. Control CR19 VV (wild-type vaccinia) was kindly provided by J. Yewdell and J. Bennink (NIAID, NIH, Bethesda, MD). Murine IL-2 cDNA

was amplified by PCR from pBMGNomIL-2 and ligated into the *SmaI-BamHI* site of a vaccinia expression vector, pMJ601 (a gift from B. Moss), which contains the β-gal gene under the control of the $p_{7.5}$ early vaccinia promoter (Whitman, E.D. et al, Surgery 116:183, 1994). The other cytokines (GM-CSF, IFN-γ, and TNF-α) were inserted into the wtVV genome with a similar procedure. The cytokines produced after infection with the rVV have been confirmed for their bioactivity.

The POXVAX-TC strain of FPV was used in these studies and is designated FPV.wt. Foreign sequences were inserted into FPV by homologous recombination as described by Jenkins, S.L. et al, AIDS Res. Hum. Retroviruses 7:991, 1991. FPV.bg40K is a recombinant that contains the *E. coli lacZ* gene under the control of the VV 40k promoter, placed in the *BamHI* J region of the FPV genome.

Peptides

The synthetic peptide, TPHPARIGL, spanning amino acids 876-884 of β-gal, the naturally processed H-2 L^d-restricted epitope (Gavin, M.A. et al, J. Immunol. 151:3971, 1993), was synthesized by Peptide Technologies (Washington, D.C.) to a purity of greater than 99% as determined by HPLC and amino acid analysis.

Evaluation of primary response

Primary lymphocyte populations were obtained by injecting 8- to 12-wk-old female BALB/c mice (Animal Production Colonies, Frederick Cancer Research Facility, National Institutes of Health, Frederick, MD) i.v. with varying PFUs of recombinant viruses. The spleens were taken on day 6 after immunization, separated into a single cell suspension and tested for their ability to lyse β-gal-positive targets in a 6 $h^{51}Cr$ release assay. Splenocytes were resuspended in complete media including RPMI 1640 with 10% FCS (Biofluids, Rockville, MD), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (Biofluids) and 5 x 10^{-5} μM2-ME (Life Technologies, Inc., Rockville,

MD), and used in 6-h_{51}CR release assays as described elsewhere (Restifo, N.P. et al J. Immunol. 147:1453, 1991). To summarize, 2 x 10^6 target cells were treated with 200 mCi $Na^{51}CrO_4$ (^{51}Cr) for 90 min. Peptide-pulsed CT26.WT were incubated with 1 μg/ml of peptide during labeling. Target and effector cells were mixed at appropriate ratios for 6 h. The amount of ^{51}Cr released was determined by gamma-counting and the percent specific lysis was calculated from triplicate samples according to the following formula:

[(experimental cpm - spontaneous cpm)/
(maximal cpm - spontaneous cpm] x 100.

Detection of cytokine production

Cytokine production was determined by ELISA. The ELISA kits for detection of murine GM-CSF, IFN-γ, TNF-α, and IL-2 from Endogen (Endogen, Boston, MA) were used according to the manufacturer's instructions. IL-2 concentrations were sometimes given in Cetus units, 1 Cetus unit/ml corresponding to 600 pg/ml of IL-2.

In vivo treatment studies

BALB/c mice were immunized with virus (5 x 10^6 to 10^7 PFUs) 3 or 6 days after i.v. challenge with tumor cells (10^5 to 5 x 10^5) to establish pulmonary metastases. All animals were randomized before receiving virus. Treatment with IL-2 was initiated 12 h after immunization; 6 doses of high dose IL-2 (100,000 Cetus units/injection) or 10 doses of low dose IL-2 (15,000 Cetus units/injection) were administered to selected groups of mice. Mice were killed on day 12 and lung metastases were enumerated in a blind fashion. Identically treated groups of mice were followed for survival.

Whole organ X-gal staining

Lungs from mice treated with i.v. tumor challenge as described above were inflated with PBS (Biofluids) before X-gal staining. Lungs were fixed in a solution containing

2% formaldehyde (v/v), 0.2% glutaraldehyde (v/v) in PBS for 45 min, washed in PBS three times, and stained in X-gal solution for 12 h at 37°C. X-gal solution for whole organs was prepared by combining the following: 0.02% (v/v) Nonidet P-40, 0.01% (w/v) sodium deoxycholate, 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ in PBS. After staining in X-gal solution, the lungs were rinsed briefly with 3% (v/v) dimethyl sulfate, and then with PBS. The stained lungs were stored at 4°C in 0.02% (w/v) sodium azide in PBS (Lin, W.C. et al, Cancer Res. 50:2808, 1990).

Statistical analysis

The Wilcoxon-Mann-Whitney U test was used to examine the null hypothesis of identify of ranks between two sets of data. Kaplan-Meier plots and Mantel-Haenszel test were used to compare survival of mice belonging to different treatment groups.

Example 27

Administration of Exogenous IL-2 with rVV encoding the model TAA reduces the number of pulmonary metastases and prolongs survival

When inoculated i.v. at a dose of 5×10^5 cells, both the parental line CT26.WT and the subclone CT26.CL25, transduced with a retrovirus encoding the model TAA β-gal, grew progressively and killed the animals in 11 to 15 days. At the time of death, these mice had greated than 500 pulmonary metastases. In the experiment presented in Table 8, control mice received no treatment and had pulmonary metastases that were too numerous to count. Neither the inoculation of moderate doses of rIL-2 for 5 days nor one i.v. injection of 5×10^6 PFU/mouse of a rVV-encoding β-gal (VJS6) induced a significant reduction of the number of pulmonary metastases counted 12 days after tumor inoculation. Immunization with VJS6 3 days after tumor injection combined with rIL-2 administration induced a significant reduction in CT26.CL25 pulmonary metastases

(p = 0.005) whereas no significant decrease in the number of pulmonary metastases of the TAA-negative parental line was seen.

Table 8

Treatment of Established Pulmonary Metastases with rVV and exogenous IL-2[a]

	Mice Inoculated With			
	CT26.WT		CT26.CL25	
rVV Treatment	Average no. metastases	Metastases/Mouse	Average No. metastases	Metastases/Mouse
None[b]	>500	>500 x 5	>500[c]	>500 x 5
VJS6	>500	>500 x 5	405.2	>500 x 3, 267, 259
Exogenous rIL-2	>500	>500 x 5	>500	>500 x 5
Exogenous RIL-2 + VJS6	406.4	>500 x 3, 298, 234	20.8	43, 6, 0, 52, 3

[a] Five BALB/c mice per each treatment group were injected i.v. with 0.5 ml of HBSS containing 5 x 10^5 tumor cells of either CT26.WT or CT26.CL25. Three days later they received a single i.v. injection of 5 x 10^6 PFU of β-gal expressing rVV, VJS6. Treatment with exogenous rIL-2 (15,000 Cetus U, twice a day, i.p.) was started 6 h after rVV inoculation and continued for 5 days. Lungs were harvested on day 12 after tumor inoculation and pulmonary metastases were counted in a blind fashion.

[b] Control mice were injected with HBSS alone.

[c] All the mice in this group died between days 11 and 12 (before lung harvest).

Active immunotherapy with the combination of exogenous rIL-2 and rVV also prolonged the survival of mice bearing 3-day-old pulmonary metastases (Figs. 22a and 22b). Two administration regimes for IL-2 were chosen: 100,000 U rIL-2 for 3 days (high dose) and 15,000 U for 5 days (low dose) were administeredd i.p. following a single i.v. injection of VJS6. As an additional negative control, a group of mice was treated with the highest dose of exogenous rIL-2 together with a TK-disrupted rVV expressing a protein different from β-gal, the influenze virus NP (V69; Figs. 22a and 22b). VJS6 exerted a marginal effect on mouse survival, consistent with a partial reduction of the number of metastases observed in some experiments (see below). A clear improvement of

survival was obtained when exogenous rIL-2 was administed with the β-gal-encoding rVV but not with the NP-expressing rVV (Fig. 22b). There was no significant difference in the survival of mice receiving the "high dose" or "low dose" rIL-2 regimens (p = 0.231). Improved survival of mice bearing the parental cell line was not observed in any treatment group (Fig. 22a).

The therapeutic advantage of exogenous rIL-2 was not limited to fully replication-competent viruses because a prolongation of survival was also observed with rFPV encoding β-gal but not with the wild-type virus (Figs. 23a and 23b). The effect of a single i.v. injection of 10^7 PFU of the recombinant FPV.bg40k, expressing β-gal under the control of the 40k vaccinia promoter, on the survival of mice bearing the β-gal-positive tumor was limited and consistent with a partial reduction in the number of pulmonary metastases. However, daily inoculation of high IL-2 doses significantly increased the therapeutic effect, resulting in 40% of mice still surviving 2 mo after treatment.

To examine a more advanced disease model, mice were injected with 10^5 tumor cells and treated on day 6. Following the i.v. inoculation of 10^5 CT26.CL25 tumor cells, all the untreated mice died by day 22 (Fig. 24). Lungs examined after 6 days of tumor growth revealed the presence of more than 100 macroscopically visible nodules (not shown). At this time point, a single inoculation of VJS6 was able to slightly increase survival, but all the mice died within 24 days. Addition of high rIL-2 dose treatment resulted in a significant survival benefit (p = 0.005) also in this advanced disease model, with two of five mice surviving until day 35. Once again, the response was specific and limited to the combination of β-gal-positive tumor and rVV; the survival of CT26.WT tumor-bearing mice was not affected (data not shown).

Example 28

Therapeutic efficacy of IL-2 produced from drVV also expressing the model TAA

The large vaccinia genome can host up to 25 kb of exogenous DNA, allowing the expression of different heterologous proteins in addition to the model TAA (Moss, B. Curr. Opin. Genet. Dev. 3:86, 1993; Mackett, M. et al. Proc. Natl Acad. Sci. USA 79:7415, 1982). The results obtained with exogenous rIL-2 prompted us to investigate the effect of cytokine production at the site of virus infection and replication. A drVV co-encoding the model TAA β-gal and IL-2 was previously described (Flexner, C. et al, Vaccine 8:17, 1990). On the basis of the above results with passively administered IL-2, a new set of dr VV in which cytokine production was driven by a powerful synthetic promoter (Davison, A.J., Nucleic Acids Res. 18:4285, 1990) was constructed. The murine cytokines included in this study were the following: IL-2, GM-CSF, IFN-γ, and TNF-α. Because the same plasmid was used to construct the various TK⁻drVV, a similar level of β-gal enzymatic activity was detected after infection of the BSC-1 cell line (data not shown). Supernatants from the same infected cultures were harvested at various times and tested for the presence of the different cytokines. Table 9 shows that specific and elevated cytokine production was detected only in supernatants from BSC-1 cells infected with the relevant viruses. For example, approximately 40,000 U IL-2/ml were released during 36 h in supernatant from BSC-1 at a moi of 1. IL-2 was barely detectable in all the other supernatants. Comparable levels of IL-2 production in murine and human tumor cell lines infected with the same rVV were recently reported (Whitman, E.D. Surgery 116:183, 1994). No cytokine was present in supernatants from cell cultures infected with the VJS6 virus expressing the E6 protein from HPV16 in addition to β-gal.

Table 9

Detection of cytokine production following in vitro infection of BSC-1 cells with 1:1 moi of different rVV[a]

rVV	IL-2	GM-CSF	IFN-γ	TNF-α
Cytokine production after 12 h				
VJS6	<34	<15	<47	<30
IL-2	2×10^6	<15	<47	<30
GM-CSF	<34	5.1×10^5	<47	<30
IFN-γ	<34	<15	5.6×10^4	<30
TNF-α	<34	<15	<47	2.05×10^3
Cytokine production after 36 h				
VJS6	<34	<15	<47	<30
IL-2	3.9×10^6	<15	<47	<30
GM-CSF	54	4.2×10^6	<47	<30
IFN-γ	94	<15	6.7×10^4	<30
TNF-α	<34	2.2×10^{3b}	<47	1.35×10^5

[a] Duplicate wells of 10^5 BSC-1 cells were infected with different rVV at a multiplicity of infection (moi) of 1 PFU/cell. At different intervals, supernatants were removed, centrifuged to eliminate cellular debris, serially diluted, and used to estimate cytokine production by using an ELISA assay specific for the murine cytokines indicated in the table. Values are expressed as pg/ml of supernatant.

[b] Only one of the two wells was positive. No positivity was detected in subsequent determinations.

When a single i.v. injection of 5×10^6 PFU/mouse was used to treat mice bearing 3-day-old pulmonary metastases, only the IL-2 drVV was able to significantly reduce the number of pulmonary nodules in mice inoculated with the β-gal-positive tumor cell line ($p \approx 0.005$) (Fig. 25b). A partial decrease was obtained with the other cytokine-encoding viruses and with VJS6. No appreciable change was observed in the number of metastases of the parental tumor cell line (Fig. 25a). The effect on pulmonary metastases also correlated with an increased survival (see below).

These resuls were particularly interesting because the drVV expressed 20- to 50-fold less β-gal enzyme activity than the VJS6 rVV, as detected after infection of BSC-1 cells with an equivalent moi (data not shown) and

also suggested that the production of IL-2 could overcome the lower production of the model TAA. When compared under the same experimental conditions, IL-2 drVV together with exogenous IL-2 gave the best results in terms of prolongation of survival ($p = 0.002$), whereas the survival in the group treated with IL-2 rVV or the combination VJS6 plus exogenous IL-2 was similar (Figs. 26a and 26b).

Example 29

IL-2 enhances the primary CTL response against VV and heterologously expressed β-gal

Because the presence of $CD8^+$ lymphocytes recognizing tumor determinants has been associated with an antitumor effect (Greenberg, P.D., Adv. Immunol. 49:281, 1991), the generation of effector CTL against β-gal in the spleens of mice 6 days after immunization was studied. This time point represents the peak of the $CD8^+$ CTL anti-vaccinia response (Bennink, J.R., Curr. Top. Microbiol. Immunol. 163:153, 1986). BALB/c mice were immunized with different rVV indicated in Figure 27, and the spleens of two mice, removed 6 days after immunization, were pooled and tested directly in a short term ^{51}Cr release assay. The response of VJS6-immunized splenocytes against the CT26.WT target cells infected with the crude 19 VV can be assumed to be the baseline for the primary response elicited by a TKVV, because the viruses presented in Figure 27 were all generated through recombination in the TK region of the vaccinia genome. The virus-driven cytokine production resulted in either an increase (IL-2 and GM-CSF rVV) or in a suppression (TNF-α and IFN-γ) of the primary cytotoxic response to vaccinia Ags. This effect was even more pronounced when a comparison between the lytic units in each spleen was performed because the difference in spleen size among the different groups; in fact, the number of lytic units in GM-CSF and IL-2 groups were increase 8.3- and 6.4-fold, whereas TNF-α and IFN-γ were reduced 5.1- and 2.9-fold, respectively, compared with the control

cytokine-negative VJS6 (Fig. 27). In the same experiments, the primary response against β-gal-positive target cells was evaluated but no lysis was detected, with the exception of a small response (5 to 10% specific ^{51}Cr release) in mice immunized with IL-2 drVV (see below). After in vitro restimulation with the antigenic peptide from β-gal (TPHPARIGL), splenocytes of BALB/c mice immunized 14 days before with 5 x 10^6 PFU of different drVV generated effector CTL able to specifically recognize the β-gal-positive clone CT26.CL25 or the parental cell line pulsed with the minimal determinant antigenic peptide. Despite the clear effect of IL-2, either exogenously administered or endogenously produced by rVV, in the treatment of tumor-bearing mice, no difference in the cytolytic activity against the specific target cells was observed in secondary cultures from animals immunized with different rVV co-expressing the TAA with the different murine cytokines (data not shown).

Because analysis of primary and secondary responses did not show any clear difference in reactivity against β-gal in normal mice inoculated with different rVV, the possibility that the in vivo response could be influenced by the presence of growing tumor by comparing the primary cytotoxic activity in normal and in tumor-bearing mice was investigated. Groups of three mice were mock inoculated (HBSS) or injected with different doses (5 x 10^5, 10^5, 5 x 10^4) of either the parental CT26.WT or the β-gal-positive CT26.CL25 cell line. After 3 days mice received a single i.v. dose of different rVV, and their spleens, collected after 6 days from virus immunization, were tested for primary CTL activity. Figure 28 summarizes the results of one experiment representative of three others. As previously indicated, immunization of normal BALB/c mice with IL-2 drVV produced a small but consistent primary response against the β-gal peptide-pulsed CT26.WT and the transduced CT26.CL25 target cells; this response was

augmented in a dose-dependent fashion in mice bearing an increasing number of β-gal-positive pulmonary metastases as a consequence of an increased inoculum of tumor cells. Conversely, the specific anti-β-gal response in mice injected with the highest dose of CT26.WT tumor cells was not comparably increased (in Fig. 28 the mice inoculated with the highest number of CT26.WT cells were shown) and was characterized by an elevated level of nonspecific cytotoxicity against the E22 target cells, a β-gal-positive line expressing a different restriction element ($H\text{-}2^b$). No response was observed in mice inoculated with 5×10^5 CT26.CL25 cells and treated with VJS6 (Fig. 28) or drVV expressing GM-CSF, IFN-γ, or TNF-α (now shown). The same enhancement of primary response was obtained with exogenous IL-2 and VJS6 (data not shown).

Example 30

Pulmonary metasases in mice dead after a prolonged survival do not present detectable levels of the model TAA

Tumor cells devoid of the β-gal marker protein are easily detected by a simple assay, facilitating the recognition of antigenic modulation occurring in vivo. Lungs from mice found dead during the survival follow-up were inflated, fixed, and exposed to an X-gal solution allowing the staining of the whole organ (Lin. W.C. et al, Cancer Res. 50:2808, 1990). Lungs from untreated mice, dead after 12 to 16 days from the i.v. injection of 5×10^5 CT26.CL25 tumor cells, showed an intense, dark-blue staining. Lungs removed from mice treated with the combination high dose exogenous rIL-2 and rVV and found dead after 50 days of survival in the experiment described in Figs. 22a and 22b were completely negative for X-gal staining as were the mice inoculated with the β-gal-negative parental cell line (data not shown). The only slight positivity detected in one sample was associated with the mouse liver, strongly adherent to the lung through a β-gal-negative invasive metastasis. This

positivity is probalby related to the β-gal-like activity present in lysosomal compartment (Sanes, J.R. et al, EMBO J. 5:3133, 1986)). A similar negativity to X-gal staining was observed in pulmonary metastases from mice treated with the combination IL-2-rVV and exogenous rIL-2 in the survival study presented in Figs. 26a and 26b.

Example 31

The effects of recombinant IL-10 on the therapeutic effectiveness of replicating and non-replicating poxviruses

BALB/c mice (5-10/group) were injected intravenously with 5 x 10^5 CT26-β-gal transfectants (CT-26.C.25) then randomized. Three days later, mice were treated with a recombinant vaccinia virus (designated VJS6) that encodes the model tumor antigen (full-length β-galactosidase) at 10^6 and 10^7 PFU either alone or in combination with 0.1μg of IL-10 I.P. per day for 7 days starting 12 hrs after the virus was given. The source of IL-10 was human recombinant IL-10 obtained from Biological Response Modifiers Program (Frederick, MD). As designated, other groups of mice were treated with a non-replicating recombinant fowlpox virus that also encodes the model tumor antigen (full-length β-galactosidase) at 10^6 and 10^7 PFU either alone or in combination with 0.1μg of IL-10 I.P. per day for 7 days starting 12 hrs after the virus was given. Pulmonary metastases were enumerated on day 12 in a coded, blinded fashion. The data is provided in Table 10.

Table 10

Adjuvant Therapy in a replicating and non-replicating setting

Group	Avg # Mets	Breakdown	P2
No Tx	250	tntc*5	---
VJS6 10e6	198	tntc*3,135,95,208	.05 (vs. no tx)
VJS6 10e6 + IL10	92	45,70,85,74,112,125,130,90	.0036 (vs. VJS6)
VJS6 10e7	49	44,40,41,84,41,40,55	.0028 (vs. no tx)
VJS6 10e7 + IL10	28	52,35,3,20,31	.005 (v. VJS6)
IL-10 alone	250	tntc*5	---
FPV 10e6	83	84,89,40,94,84,65,49,61,125	.002 (vs. no tx)
FPV 10e6 + IL10	20	15,4,6,8,0,12,40,82	.024 (v. FPV)
FPV 10e7	10	0*2,3,3,5,2,15,12,49	.002 (vs. no tx)
FP 10e7 + IL10	24	0,0,2,4,29,30,55,38,45,46	> .05

In another experiment BALB/c mice (5-10/group) were injected intravenously with 5 x 10^5 CT26-β-gal transfectants (CT-26.C.25) then randomized. Three days later, mice were treated with number of PFU listed on the x-axis of a recombinant vaccinia virus (designated VJS6) that encodes the model tumor antigen-full-length β-galactosidase either alone or in combination with 0.1μg of IL-10 per day for 7 days starting 12 hrs after the virus was given. Pulmonary metastases were enumerated on day 12 in a coded, blinded fashion. Animals receiving no treatment or IL-10 alone had an average of 250 pulmonary metastases (Figure 29).

In yet another experiment, mice were injected I.V. with 5 x 10^5 CT26-β-gal transfectants. Three days later mice were treated with 10^1, 10^2, 10^3, 10^4, 10^5, 10^6, or 10^7 PFU of VJS6 either alone or in combination with 0.1μg of

IL-10 per day for 7 days starting 12 hrs after the virus was given. Pulmonary metastases were enumerated on day 12 as above. Animals receiving no treatment or IL-10 alone had an average of 250 pulmonary metastases (Figure 30).

While the invention is described above in relation to certain specific embodiments, it will be understood that many variations are possible, and that alternative materials and reagents can be used without departing from the invention. In some cases such variations and substitutions may require some experimentation, but will only involve routine testing.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and therefore such adaptations and modifications are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments.

All references and patents referred to are incorporated herein by reference.

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What is claimed is:

1. A recombinant virus comprising a recombinant virus which has incorporated into a viral genome or portion thereof one or more genes or portion thereof encoding an antigen of a disease causing agent and one or more genes or portions thereof encoding an immunostimulatory molecule.

2. The recombinant virus according to claim 1 where the virus is nonpathogenic.

3. The recombinant virus according to claim 1 wherein the virus is nonreplicating.

4. The recombinant virus according to claim 1 wherein the virus is selected from the group consisting of retrovirus, baculovirus, Ankara virus, fowlpox, adenovirus, and vaccinia virus.

5. The recombinant virus according to claim 1 wherein the disease causing agent is a cancer or pathogenic microorganism.

6. The recombinant virus according to claim 5 wherein the cancer is a non-Hodgkin Lymphoma, leukemia, Hodgkins lymphoma, lung cancer, liver cancer, metastases, melanoma, adenocarcinoma, thymoma, colon cancer, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer or sarcoma.

7. The recombinant virus according to claim 5 wherein the pathogenic microorganism is virus, bacterium, protozoan, or yeast.

8. The recombinant virus according to claim 7

wherein the pathogenic virus is HIV, hepatitis virus, human papillomavirus, equine encephalitis virus, herpes simplex virus or influenza virus.

9. The recombinant virus according to claim 6 wherein the antigen is a tumor associated antigen.

10. The recombinant virus according to claim 9 wherein the tumor associated antigen is selected from the group consisting of oncofetal antigens, MART-1, Mage-1, Mage-3, gp100, tyrosinase, CEA, PSA, CA-171A, CA-19-A, CA-125, erb-2, TRP-1, P-15 and β-galactosidase.

11. The recombinant virus according to claim 1 wherein the immunostimulatory molecule is selected from the group consisting of IL-2, B7.1, B7.2, ICAM-1, LFA-3, CD72, GM-CSF, TNFα, IFNγ, IL-10, IL-12, IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-13, IL-14, IL-15, ICAM-2, LFA-1, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B, RANTES and combinations thereof.

12. The recombinant virus according to claim 11 comprising the immunostimulatory molecule, IL-2 and B7.1.

13. The recombinant virus according to claim 1 wherein the recombinant virus is vaccinia virus.

14. The recombinant virus according to claim 13 wherein the gene encoding the antigen is incorporated into a HIND F13L region or TK region of the vaccinia virus.

15. The recombinant virus according to claim 13 wherein the gene encoding the immunostimulatory molecule is incorporated into a HIND F13L region or HA region of the vaccinia virus.

16. A recombinant virus comprising a recombinant vaccinia virus which has incorporated into a genome a gene encoding a tumor associated antigen and a gene encoding an immunostimulatory molecule.

17. The recombinant virus according to claim 16 wherein the tumor associated antigen is an oncofetal antigen, MART-1, Mage-1, Mage-3, gp100, tyrosinase, CEA, PSA, CA-171A, CA-19-A, CA-125, erb-2, TRP-1, P-15 or β-galactosidase.

18. The recombinant virus according to claim 16 wherein the immunostimulatory molecule is ICAM-1, LFA-3, CD72, GM-CSF, TNFα, IFNγ, IL-12, RANTES, IL-10, IL-2, B7.1, B7.2, IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-13, IL-14, IL-15, ICAM-2, LFA-1, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B or combinations thereof.

19. A method of preventing a disease in a mammal comprising administering to the mammal an effective amount of a recombinant virus according to claim 1, alone or in combination with an exogenous immunostimulatory molecule said amount is effective in preventing or ameliorating said disease.

20. The method according to claim 19, wherein the disease is caused by a cancer or pathogenic microorganism.

21. The method according to claim 19, wherein the cancer is non-Hodgkin lymphoma, Hodgkins lymphoma, leukemia, lung cancer, liver cancer, metastases, melanoma, adenocarcinoma, thymoma, colon cancer, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer or sarcoma.

22. The method according to claim 19 wherein the pathogenic microorganism is an intracellular virus, bacterium, protozoan, or yeast.

23. The method of claim 19 wherein the exogenous immunostimulatory molecule is IL-2, GM-CSF, TNFα, TNFγ, IL-12, RANTES, B7-1, B7-2, ICAM-1, LFA-3, IL-10, CD72, IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-13, IL-14, IL-15, ICAM-2, LFA-1, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B or combinations thereof.

24. A pharmaceutical composition comprising the recombinant virus according to claim 1 alone or in combination with an exogenous immunostimulatory molecule, chemotherapy drug, antibiotic, antifungal drug, antiviral drug or combination thereof and a pharmaceutically acceptable carrier.

25. The pharmaceutical composition according to claim 24 wherein the exogenous immunostimulatory molecule is ICAM-1, LFA-3, CD72, GM-CSF, TNFα, IFNγ, IL-10, IL-12, RANTES, IL-2, B7.1, B7.2, IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-13, IL-14, IL-15, ICAM-2, LFA-1, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B or combination thereof.

26. A method of making an immune-enhancing recombinant virus against a disease causing agent comprising inserting a nucleic acid sequence encoding one or more antigens or immunodominant epitopes thereof and a nucleic acid sequence encoding one or more immunostimulatory molecules into the genome or portion thereof of a recombinant virus.

27. An immune-enhancing recombinant virus made

according to the method of claim 26.

28. A pharmaceutical composition comprising a recombinant virus, one or more exogenous immunostimulatory molecules and a pharmaceutically acceptable carrier, said virus having incorporated into a viral genome or portion thereof one or more genes or portion thereof encoding an antigen of a disease causing agent.

29. The pharmaceutical composition according to claim 28 wherein the disease causing agent is a cancer or a pathogenic microorganism.

30. The pharmaceutical composition according to claim 28 wherein the antigen is a tumor associated antigen.

31. The pharmaceutical composition according to claim 28 wherein the exogenous immunostimulatory molecule is IL-2, GM-CSF, TNFα, IFNγ, IL-10, IL-12, RANTES, B7.1, B7.2, ICAM-1, LFA-3, IL-10, CD72, IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-13, IL-14, IL-15, ICAM-2, LFA-1, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B or combination thereof.

32. The pharmaceutical composition according to claim 30 wherein the exogenous immunostimulatory molecule is IL-2, IL-10, or combination thereof.

33. A method of preventing a disease in a mammal comprising administering to the mammal an effective amount of the composition according to claim 28, said amount is effective in preventing or ameliorating said disease.

FIG. 1

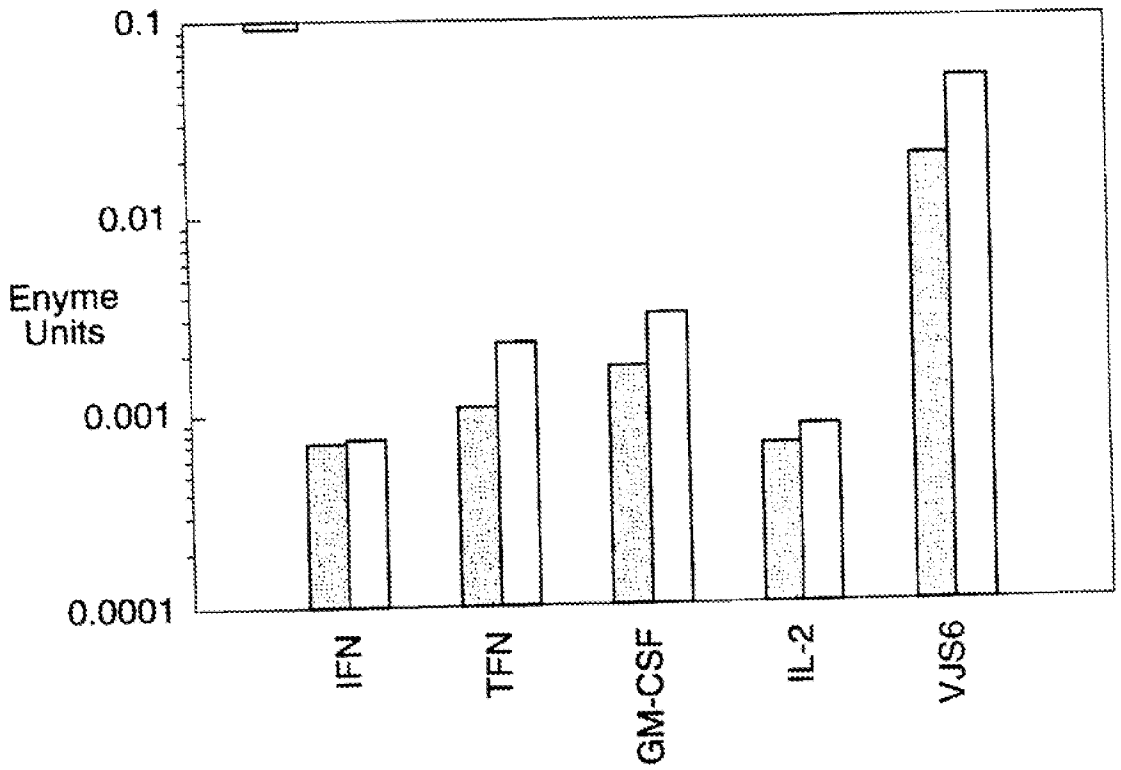


FIG. 2

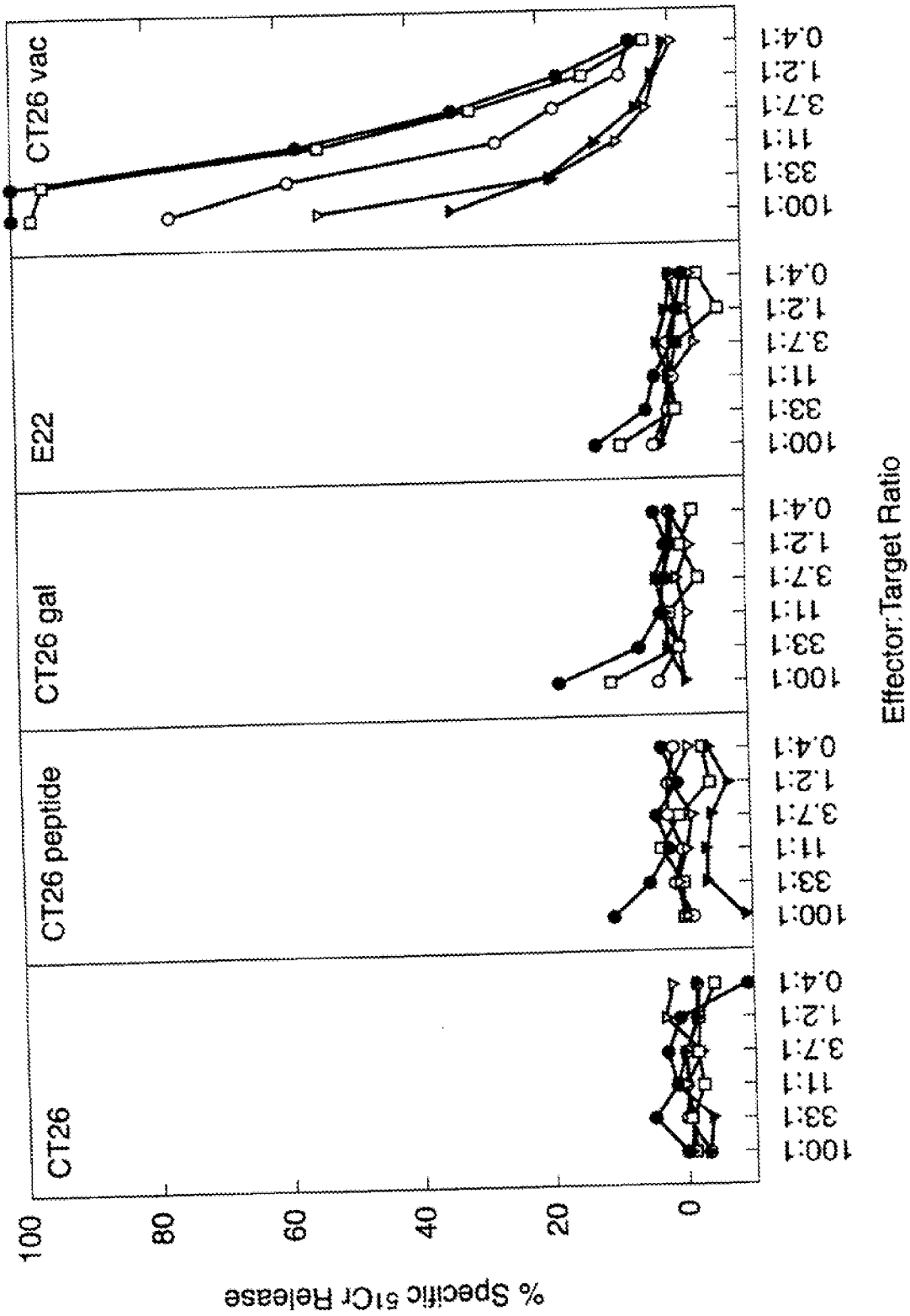

CT26 vac
E22
CT26 gal
CT26 peptide
CT26
100:1
33:1
11:1
3.7:1
1.2:1
0.4:1
Effector:Target Ratio
% Specific 51Cr Release
100
80
60
40
20
0

FIG. 3

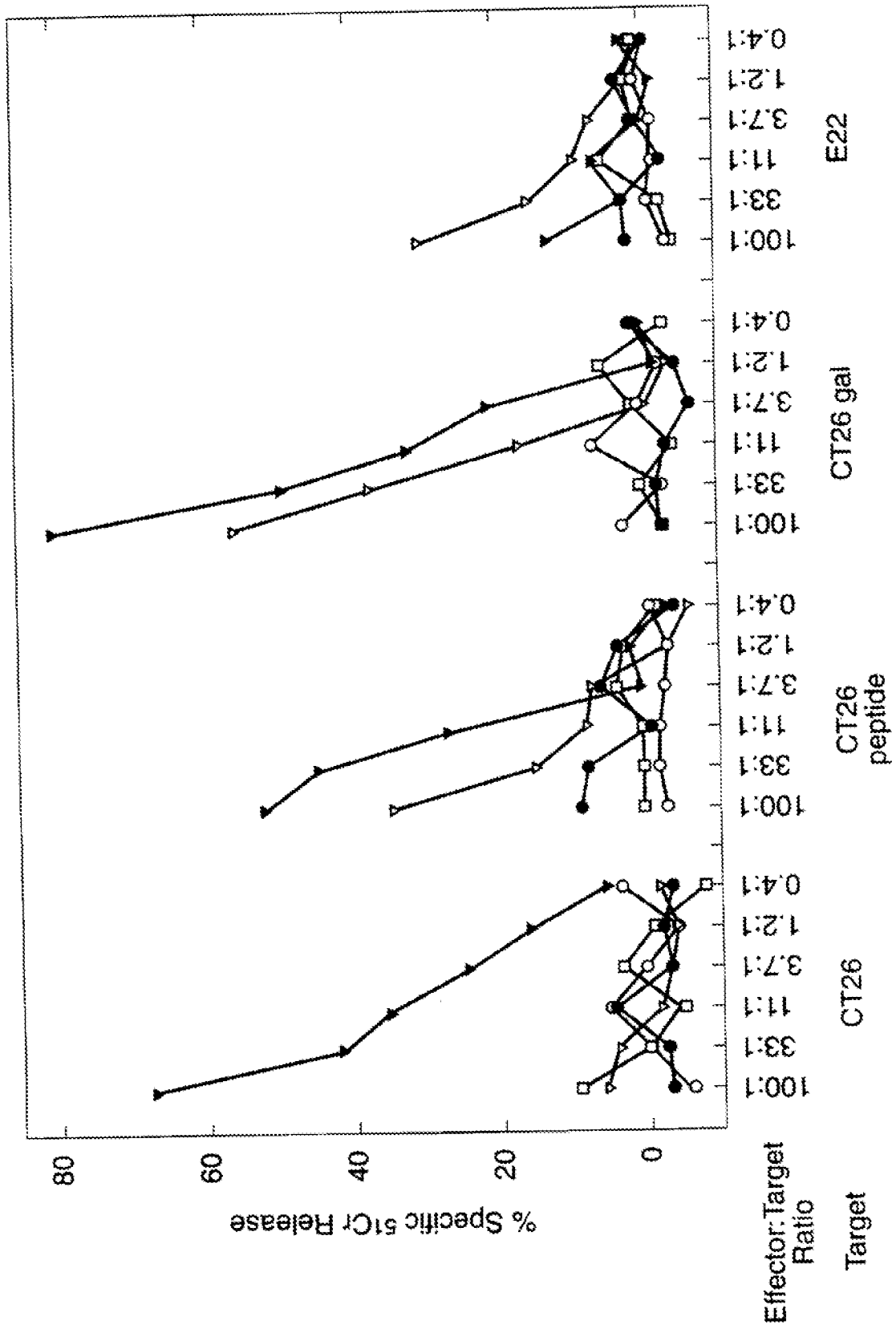

% Specific 51Cr Release
0
20
40
60
80
Effector:Target Ratio
Target
100:1
33:1
11:1
3.7:1
1.2:1
0.4:1
CT26
CT26 peptide
CT26 gal
E22

FIG. 4

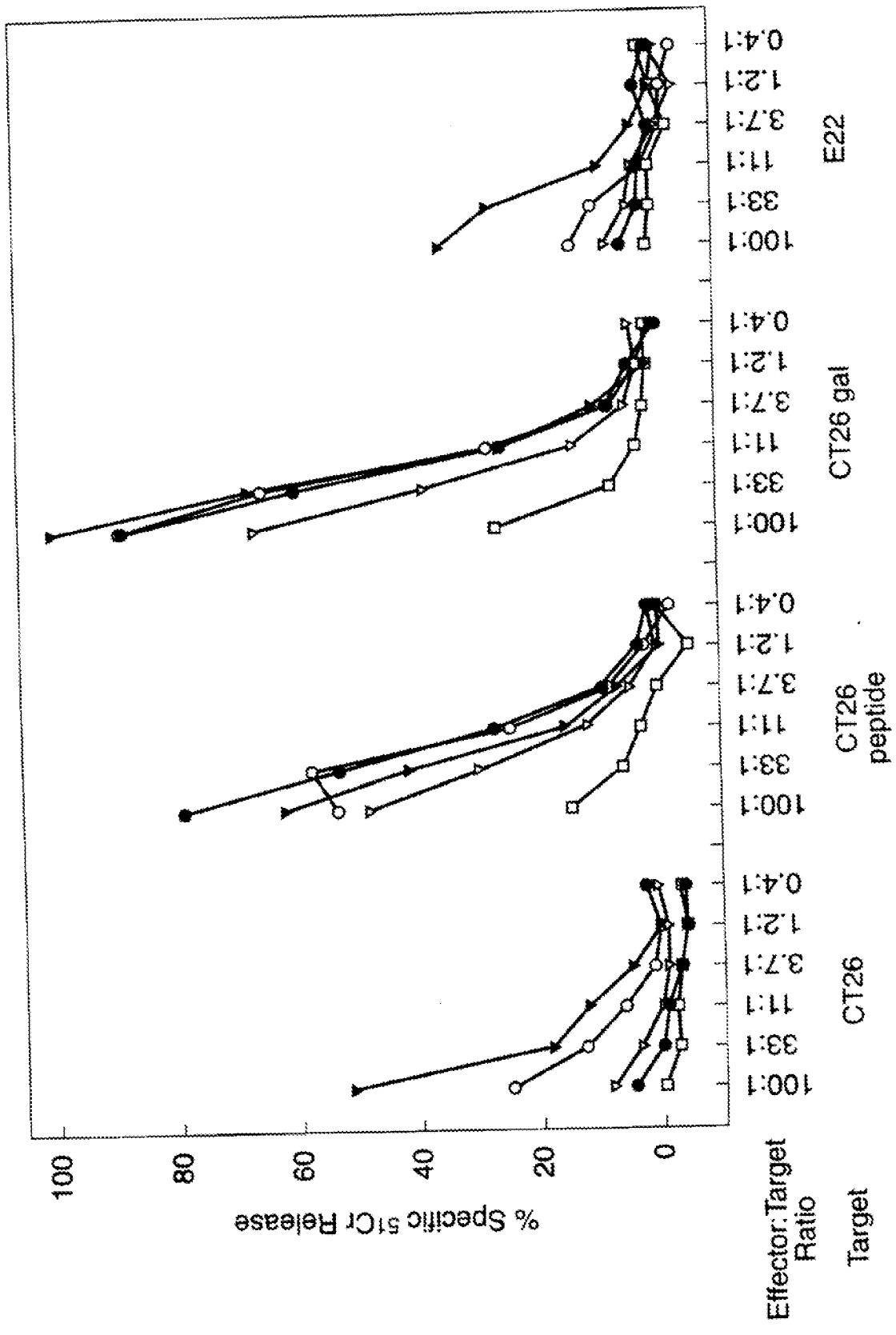

% Specific 51Cr Release
100
80
60
40
20
0
Effector:Target Ratio
Target
100:1
33:1
11:1
3.7:1
1.2:1
0.4:1
CT26
CT26 peptide
CT26 gal
E22

FIG. 6

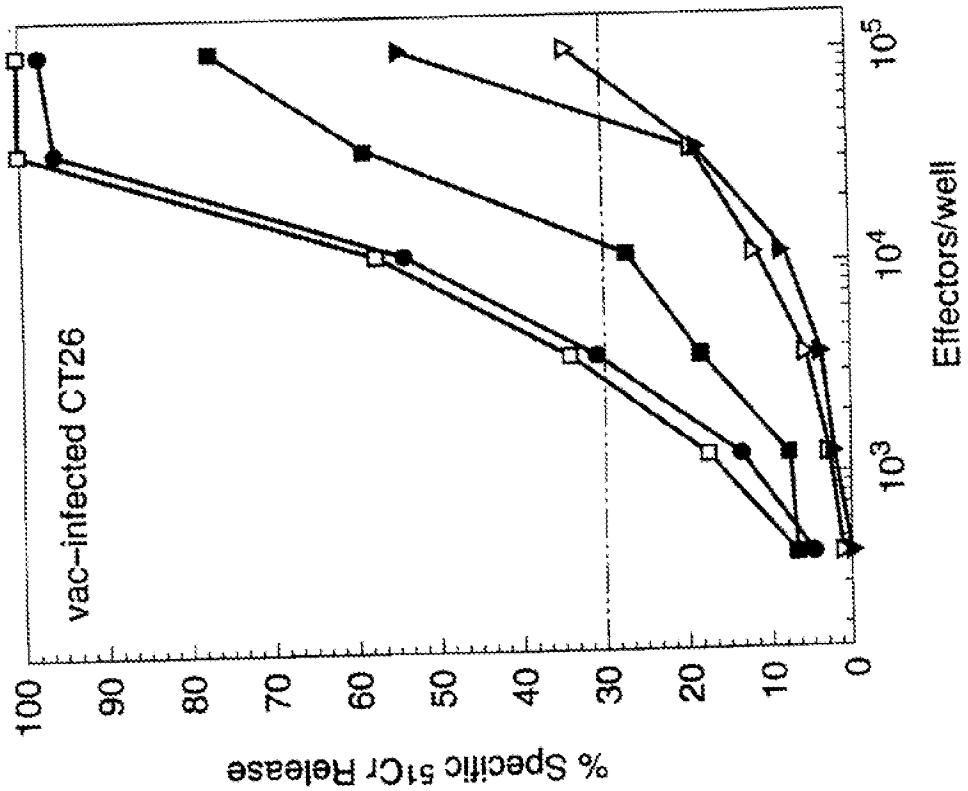
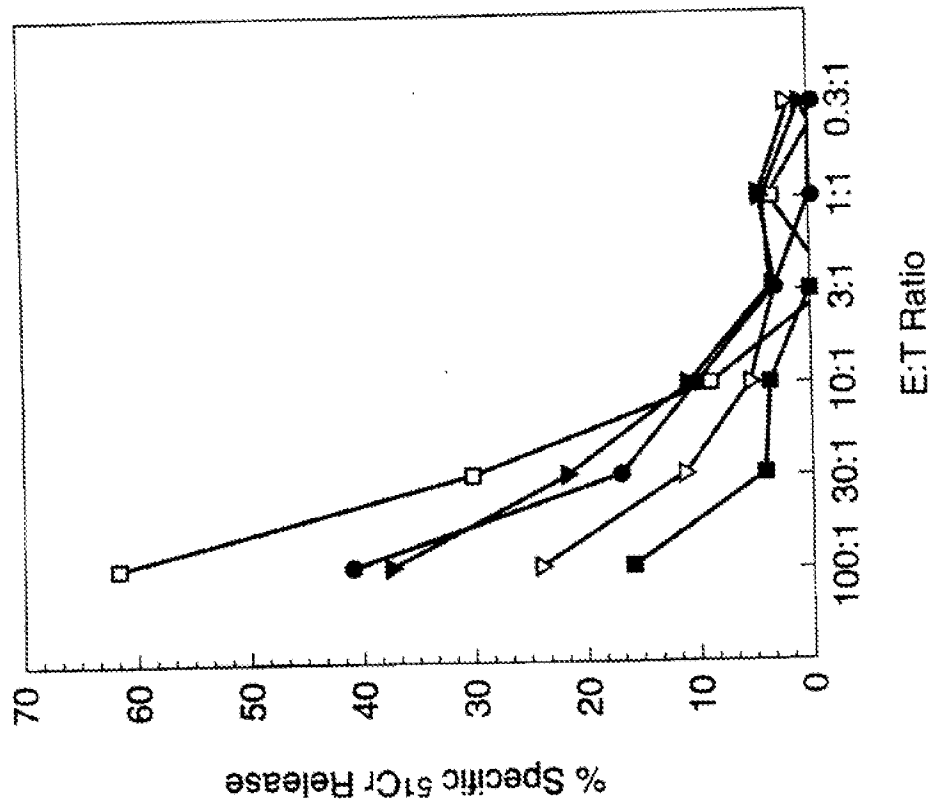

vac-infected CT26
% Specific 51Cr Release
100
90
80
70
60
50
40
30
20
10
0
Effectors/well
10^3
10^4
10^5

FIG. 5


% Specific 51Cr Release
70
60
50
40
30
20
10
0
E:T Ratio
100:1
30:1
10:1
3:1
1:1
0.3:1